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The goal of this proposal was to engineer plants to produce butanetriol and phloroglucinol, which are precursors of energetic materials butanetriol trinitrate (BTTN) and 1,3,5-triamino-2,4,6 trinitrobenzene (TATB), respectively, in plants. The strategy was to introduce bacterial genes involved in synthesis of these chemicals in to plants. Synthesis of these precursors of energetic materials in plants overcomes many problems associated with the current chemical-based methods. Bacterial synthesis of butanetriol from xylose or arabinose takes place in a pathway that requires four enzymes. We have introduced four bacterial genes involved in synthesis of butanetriol from either xylose or arabinose into plants and shown that plants can produce butanetriol (Abdel-Ghany et al., Metabolic Engineering, 20, 109-120, 2013). We have also demonstrated that introduction of bacterial phloroglucinol synthase into plants results in production both phloroglucinol and a phloroglucinol glucoside "phlorin". These proof-of-concept experiments were carried out in Arabidopsis. To introduce these pathways into Miscanthus, a non-food crop, we have developed an efficient regeneration system for this plant.

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Final Report

A. PI: Anireddy S.N. Reddy

B. Organization: Colorado State University

C. ONR Award Number: N000140810470

D. Award Title: Metabolic Engineering of Plants to Produce Precursors (Phloroglucinol and 1,2,4-butanetriol) of Energetic Materials

Abstract

The goal of this proposal was to engineer plants to produce butanetriol and phloroglucinol, which are precursors of energetic materials butanetriol trinitrate (BTTN) and 1,3,5-triamino-2,4,6 trinitrobenzene (TATB), respectively, in plants. The strategy was to introduce bacterial genes involved in synthesis of these chemicals in to plants. Synthesis of these precursors of energetic materials in plants overcomes many problems associated with the current chemical-based methods. Bacterial synthesis of butanetriol from xylose or arabinose takes place in a pathway that requires four enzymes. We have introduced four bacterial genes involved in synthesis of butanetriol from either xylose or arabinose into plants and shown that plants can produce butanetriol (Abdel-Ghany et al., Metabolic Engineering, 20, 109-120, 2013). We have also demonstrated that introduction of bacterial phloroglucinol synthase into plants results in production both phloroglucinol and a phloroglucinol glucoside "phlorin". These proof-of-concept experiments were carried out in Arabidopsis. To introduce these pathways into Miscanthus, a non-food crop, we have developed an efficient regeneration system for this plant.

Accomplishments

Metabolic engineering of plants to produce phloroglucinol

Phloroglucinol synthase (PhlD) from *Pseudomonas* has been shown to convert malonyl Co-A to phloroglucinol. In plants malonyl Co-A is produced in the chloroplast and a small amount is exported to the cytosol. We generated transgenic lines expressing either bacterial *PhlD* gene or codon-optimized synthetic *PhlD* gene directed to either cytosol or to chloroplast. Eight homozygous lines (two for each construct) were screened for phloroglucinol production.

GC-MS and LC-MS analyses of wild type and transgenic lines in which PhlD is either produced in cytosol or targeted to chloroplasts have shown a peak (peak #1) corresponding to the retention time of phloroglucinol (about 521 sec) and another peak (peak #2) at 900 sec, which could be a conjugated from of phloroglucinol or some other product. Both these peaks were not detected in wild type. The area of the second peak indicates that the second compound is much more abundant than the first one.

To further confirm that the compound in peak #1 is phloroglucinol we performed in source fragmentation of peak #1 compound. The MS/MS spectra of peak #1 matched with phloroglucinol spectra, confirming that the compound in peak #1 is phloroglucinol. These results show that plants expressing PhlD do produce phloroglucinol. To identify the compound in peak #2, we used two independent analytical platforms (LC-MS and GC-MS). In UPLC-QTOF analysis peak #2 compound is absent in the wild type. The spectra for this compound suggested a molecular weight of 287.076 and generated a prominent in-source fragment at 125.0239 m/z in negative ionization mode and 127.02 m/z in positive ionization mode. MS/MS analysis confirmed that 125.0239 spectrum is a product of 287.0764 and this signal is consistent with a conjugate of phloroglucinol, but not pyrogallol. Furthermore, the molecular weight of the compound in peak #2 suggests that it is likely a hexose conjugate of phloroglucinol. We further confirmed that Peak #2 is a conjugate of phloroglucinol using GC-MS also. In GC-MS analysis, phloroglucinol eluted at 8.64 min and the mass spectrum indicated a molecular weight of 342 m/z. In transgenic lines, in addition to phloroglucinol peak #1, peak #2 with a retention time of 14.78 min was detected only in the transgenic line, suggesting that it is a conjugated from of phloroglucinol. In addition, based on the molecular weight of this compound it appears that a hexose molecule is conjugated to phloroglucinol.

A previous study has shown that plants fed with phloroglucinol and glucose produce a phloroglucinol conjugate called phlorin (phloroglucinol beta-D-glucoside). We found that Arabidopsis leaves fed with phloroglucinol and glucose can also produce a compound with retention time similar to peak #2. To test if the peak #2 compound is phlorin, we extracted phlorin from an orange peel, a tissue know to contain high levels of phlorin, and analyzed on GC-MS. The elution time of phlorin is identical to peak #2 in our transgenic lines, suggesting that most of the phloroglucinol produced in our transgenic lines is converted into phlorin. To further confirm that the peak #2 product in our transgenic lines is phlorin, we analyzed it and

the purified phlorin from an orange peel using GC-MS/MS. The mass spectra of peak #2 compound and phlorin from orange peel were identical. Furthermore, NMR analysis of peak #2 compound also confirmed that it is phlorin. All our results confirm that the compound in peak #2 is phlorin, a phloroglucinol conjugate and that a substantial amount of phloroglucinol produced in our transgenic lines is converted into phlorin.

To quantify the amount of the phloroglucinol and its conjugate (phlorin) in the transgenic lines, we grew wild type and eight transgenic lines on soil for 2 weeks. Seedlings were harvested in triplicates, ground to powder, lyophilized and used for GC-MS analysis. We found higher accumulation of phlorin in the transgenic lines where PhlD is targeted to chloroplasts, with the highest accumulation in one of the chloroplastic lines. This line showed much reduced growth as compared to wild type and all other transgenic lines. This phenotype might be related to the depletion of malonyl CoA, a precursor for fatty acid synthesis and elongation, in chloroplasts. Since malonyl CoA could be limiting factor in phloroglucinol production, we tested the effect of exogenous malonate on phloroglucinol and phlorin accumulation. We grew wild type and one transgenic line for each construct on MS medium containing 2.5 mM malonate. We did not observe any increase in phlorin amount in the presence of malonate.

To see if there is differential accumulation of phloroglucinol and phlorin in roots and shoots, we grew plants hydroponically for 3 weeks, shoots and roots were then harvested separately and used for GC-MS analysis. Interestingly, a higher level of phlorin was found in roots as compared to shoots, especially in lines where PhlD is targeted to chloroplasts.

In addition to targeted analysis, we performed non-targeted analysis of all metabolites in wild type and transgenic lines using Principal Component Analysis (PCA). This analysis has placed wild type, transgenic lines producing PhlD in cytosol and those lines producing PhlD in chloroplasts in three distinct groups, indicating that introduction of bacterial PhlD into plants not only resulted in production of phloroglucinol and its conjugate but also altered the profile of several other metabolites. Among the metabolites that are significantly accumulated in the chloroplastic lines and partially in the cytosolic line but not in the wild type are salicylic acid, serine, purine, phenylalanine, valine and hexose sugars. On the other hand the metabolites that are completely depleted in the chloroplastic lines and partially in cytosolic lines as

compared to wild type are malic acid, oxa-glutarate, carbonic acid and sinapic acid. A manuscript describing these results will be submitted for publication.

Metabolic engineering of plants to produce butanetriol

A manuscript (*Salah E. Abdel-Ghany, Irene Day, Adam L. Heuberger, Corey D. Broeckling and Anireddy S.N. Reddy) Metabolic engineering of Arabidopsis for butanetriol production using bacterial genes*) describing our results on production of butanetriol in plants using bacterial arabinose and xylose pathways genes is published in "Metabolic Engineering" journal (http://www.biology.colostate.edu/files/2013/10/Metabolic-Engineering-Paper.pdf). Since all the data pertinent to this objective were presented in this published paper we have not summarized these results here.

To develop regeneration and robust stable transformation technologies for Miscanthus to introduce and express genes involved in synthesis of energetic materials.

We have developed highly efficient and robust methods to induce calli and to regenerate whole plants from the calli of *Miscanthus giganteus*. In our transformation trials, we have found that the untransformed *Miscanthus* calli are resistant to both kanamycin and Basta, two commonly used chemicals to select transgenic lines, but not to hygromycin, another selectable marker. Hence, we developed new constructs containing hygromycin resistance gene and reporter genes (GUS or RFP). Using either agrobacterium co-cultivation or particle bombardment under many different conditions, a total of more than 150 trials have been tested. Although we obtained calli that grew on selection and regenerated into plantlets, the molecular analysis by PCR confirmed that that they are not true transgenic lines, which is common in monocots. For example, about 10% of regenerated plants in rice transformation are false positives. Based on our trials it appears that *Miscanthus giganteus* is highly recalcitrant for transformation.